ENZYMATIC BLOCK IN HIGHER GANGLIOSIDE BIOSYNTHESIS IN AVIAN TRANSPLANTABLE LYMPHOID TUMOR

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1. Introduction

Since the initial report by Hakomori and Murikami [1] of an altered glycolipid pattern in polyoma-transformed baby hamster kidney cells, many investigators have demonstrated changes in glycolipid patterns in tumorigenically transformed cells and tissues. Brady et al. [2–4] observed accumulation of hematoside (GM3)* at the expense of higher gangliosides in several transformed cell lines. This accumulation of GM3 was subsequently shown to be due to depression in activity of the enzyme which transfers *N*-acetylgalactosamine from UDP-*N*-acetylgalactosamine to GM3 with resultant formation of GM2 [5,6]. Because of the inherent difficulties in separating effects of transformation from cloning and selection effects in cell cultures, recent attention has been focused on ganglioside alterations in solid tumors. Siddiqui and Hakomori [7] and Cheema et al. [8] have observed that disialogangliosides accumulate at the expense of trisialogangliosides in several lines of Morris hepatomas. More recently Keenan and Morré [9] found that GMl accumulates in chemically induced rat mammary carcinomas due to depression of the sialyl transferase which functions in the conversion of GMl to the GDla disialoganglioside.

Altered glycolipid patterns occurring during malignant transformation are believed to reflect membrane changes which are phenotypic manifestations of tumorigenesis. If these alterations are necessary to permit malignant growth, they should be of general occurence in tumors. To explore this possibility we have studied the synthesis and distribution of gangliosides in a transplantable avian lymphoid tumor. This tumor cell line was established in chickens in 1937 [10] and has been maintained by serial passage. This tumor is transplantable but not transmissible [11–13], although a virus (RPL12) can be isolated from the tumor which produces tumors distant to the site of innoculation after a very long incubation time [14]. The available evidence indicates that bursal lymphocytes are the cells of origin of the transplantable lymphoid tumor [15,16].

2. Materials and methods

Rapidly growing transplantable lymphoid tumours (TLT) were harvested from 5-weeks old Hubbard broiler-type chickens 10 days after inoculation. Tumors were minced in Rous–Turner saline [13] containing 10% dimethylsulfoxide, filtered, frozen and held at –60°C to provide a common pool of tumor cells used to inoculate all birds in this study. Aliquots of TLT cell inoculum were thawed and injected either intramuscularly in the right pectoral muscle or in the abdominal cavity of 4-weeks-old chickens.

* Standard abbreviations used are: GM3, sialylgalactosyl-glucosylceramide; GM2, *N*-acetylgalactosaminyl-(sialyl)-galactosylglucosylceramide; GM1, galactosyl-*N*-acetylgalactosaminyl-(sialyl)-galactosylglucosylceramide; GD3, $(sialyl)_2$ galactosylglucosylceramide; GD1a, sialyl-galactosyl-*N*-acetyl-gal actosaminyl-(sialyl)-galactosylglucosylceramide; GD1b, galactosyl-*N*-acetyl-galactosyl-$(sialyl)_2$-galactosylglucosylceramide; NAN, *N*-acetylneuraminic acid; Gal, galactose; Glc, glucose; UDP, uridine-5′-diphosphate; CMP, cytidine-5′-monophosphate; GalNAc, *N*-acetylgalactosamine. Ganglioside abbreviations are those of Svennerholm [24].

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Table 1
Composition of avian transplantable lymphoid tumor and control tissues.

Tissue	Total sialic acid[a]	Bound sialic acid[a]	Ganglioside sialic acid[a]	Total lipid[b]	Lipid phosphorus[c]
Bursa	17.54	16.76	2.34	0.22	3.50
Thymus	17.59	14.18	1.64	0.80	3.44
Muscle	8.82	6.23	0.30	0.12	0.89
IM Tumor 1	23.41	15.68	1.62	0.24	1.52
IM Tumor 2	18.72	13.93	1.47	0.27	2.64
IP Tumor 1	25.12	20.37	1.62	0.63	5.29
IP Tumor 2	38.39	32.88	2.10	0.73	6.34

[a]Nanomoles per mg protein.
[b]Mg per mg protein.
[c]Micrograms per mg protein.

The tumors, localized at the inoculation sites in the muscle (designated IM tumors hereafter) or on the serosa of the abdominal cavity (designated IP tumors hereafter), were harvested 10 days later. Tumors were dissected free of normal and necrotic tissue, minced and placed in ice-cold 0.32 M sucrose–14 mM 2-mercaptoethanol. Bursa of Frabicius, thymus and pectoral muscle were removed from control birds of the same age and each pooled, minced and placed in 0.32 M sucrose–14 mM 2-mercaptoethanol.

Samples were homogenized [17] and the protein [18] and total and bound sialic acid [19] contents were determined. Lipids were extracted and gangliosides were recovered and measured by sialic acid assay as in previous studies [17, 20]. Total lipid was determined gravimetrically and lipid phosphorus by colorimetric assay [21]. Gangliosides were separated by thin-layer chromatography on 500 μm layers of Silica gel G developed in chloroform–methanol–28% ammonia–water (60:30:7:3, by volume) and the ganglioside bands were detected with resorcinol reagent [22].

Total particulate fractions, prepared as in previous studies, served as the source of enzyme for glycolipid glycosyl transferase assays [9, 23]. These assays were performed as described previously [9, 23]. Specific activity values were calculated from the amount of radioactive sugar transferred to glycolipid. Glycolipid acceptors were prepared as described [9, 23]. Radioactive sugar nucleotides were obtained from New England Nuclear Corp. Unlabeled sugar nucleotides were either commercial preparations or gifts.

3. Results

Available evidence indicates that bursal lymphocytes are the cells of origin of lymphoid leucosis tumor cells [15, 16] and thus bursa was included as a control tissue in these studies. It could be speculated that thymus may possibly be the origin of TLT cells and for this reason thymus was also assayed. Tumors growing in the pectoral muscle (IM tumors) were invariably contaminated with muscel to the extent of about 15% as determined histologically. Pectoral muscle was assayed to assess extent to which muscle contamination of IM tumors would affect results. Two separate batches of IM and IP tumors were analyzed. Each set was obtained from a minimum of five birds. For enzyme assays, sets were combined.

Both IP tumors had higher levels of total and bound sialic acids than did IM tumors, bursa and thymus (table 1). IM tumors were similar to bursa and thymus in levels of total and bound sialic acid. No major differences in levels of ganglioside sialic acid were evident among the IM and IP tumors, bursa and thymus. Muscle contained less than half the levels of total and bound sialic acids encountered in other tissues (table 1). Only very low levels of ganglioside sialic acid were found in muscle. On a protein basis, total lipid content of IM tumors was nearly identical to total lipid levels in bursa. Bursa contained higher levels of phospholipid than did IM tumors (table 1). IP tumors contained nearly 3 times more lipid than IM tumors. IP tumors were similar

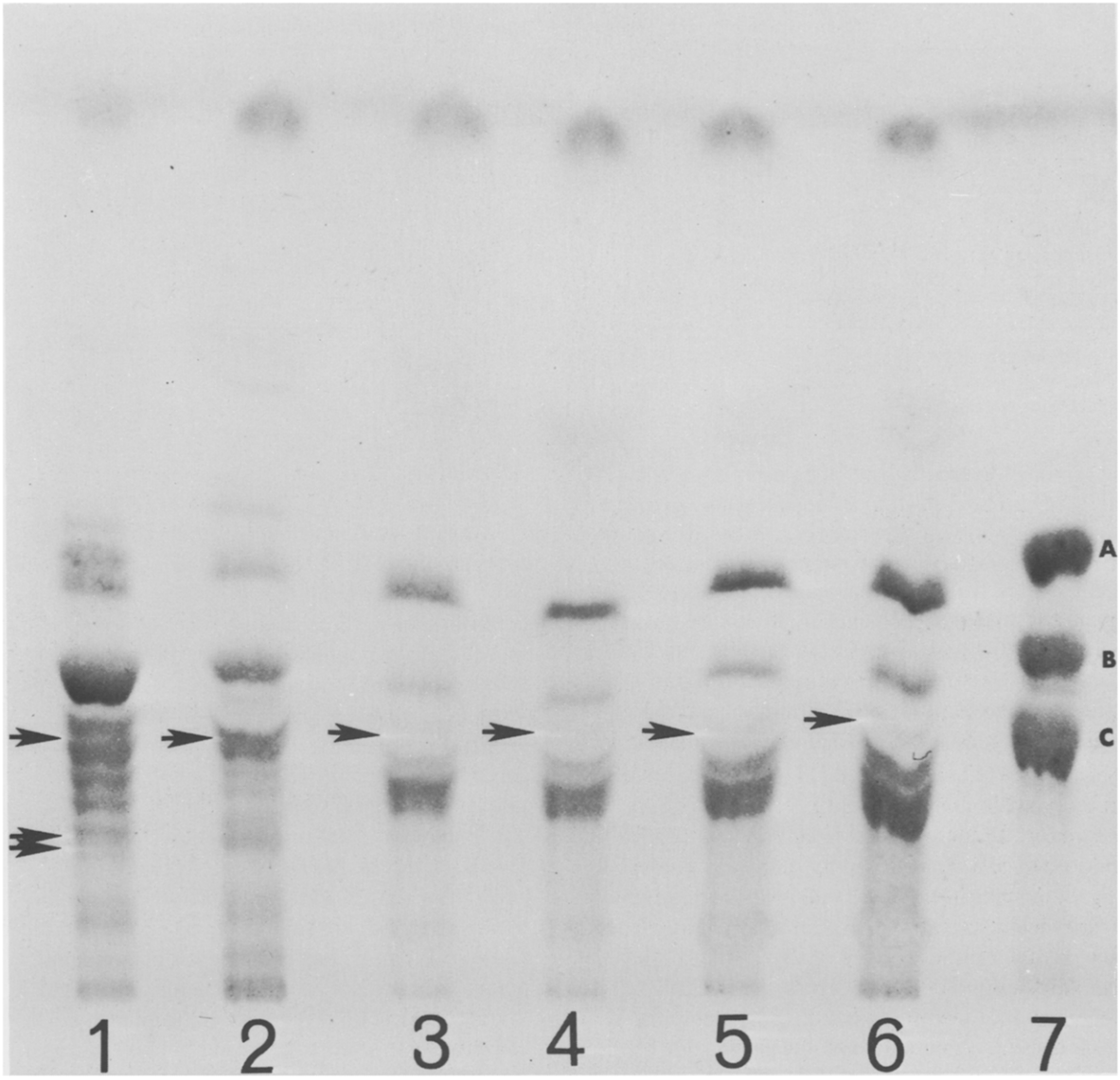

Fig. 1. Thin-layer chromatographic pattern of ganglioside fractions from avian transplantable lymphoid tumor and control tissues. 1, Bursa; 2, thymus; 3, intramuscular tumor-1; 4, intramuscular tumor-2; 5, intraperitoneal tumor-1 6, intraperitoneal tumor-2; 7, reference gangliosides GM3 (A), GM2 (B), and GM1 (C). Single arrows denote position of GM1. Double arrows denote region of trisialogangliosides. Approximately 80 nmoles of ganglioside sialic acid from each tissue was applied to the plate. The 250 μm silica gel G plate was developed in chloroform–methanol–28% ammonia–water (60:35:7:3, by volume) and was sprayed with resorcinol reagent.

to thymus in total lipid levels. Phospholipids represented a higher proportion of total lipids in IP tumors than in any of the other tissues examined. As with sialic acids, muscle contained much lower levels of total lipid and lipid phosphorus than the other tissues.

While there were no appreciable differences in total ganglioside levels in bursa, thymus and the tumors, pronounced differences were evident in ganglioside distribution patterns (fig. 1). Bursa contained gangliosides migrating with hematosides (GM3),

Table 2
Ganglioside biosynthetic enzyme activities in avian transplantable lymphoid tumor and control tissues.

Acceptor	Nucleotide sugar	Specific activity[a]				
		Bursa	Thymus	Muscle	IM Tumor	IP Tumor
Glucosyl ceramide	UDP–Gal	110.7	31.9	10.1	63.8	75.5
None	UDP–Gal	16.8	1.7	13.4	11.7	18.5
Lactosyl ceramide	CMP–NAN	147.5	302.8	4.9	40.3	75.7
GM3	CMP–NAN	41.3	78.6	9.9	14.7	22.6
GM1	CMP–NAN	198.6	115.0	6.9	33.5	54.1
None	CMP–NAN	19.6	54.1	6.9	7.8	24.6
GM3	UDP–GalNAc	306.7	329.2	8.3	240.0	194.2
None	UDP–GalNAc	15.8	8.3	0.8	14.2	18.3
GM2	UDP–Gal	472.1	192.6	6.3	36.6	57.9
None	UDP–Gal	16.3	18.9	3.8	8.8	13.9

[a]Specific activities are pmoles/mg protein/hr except for UDP–GalNAc transferase where specific activities are CPM/mg protein/hr. Reaction mixtures contained 0.4 mg particulate protein., 0.05 μmole acceptor and 0.05 μmole nucleotide sugar (0.01 μmole for UDP–GalNAc). Other constituents of the reaction mixtures are given in references [9] and [23].

GM2, GM1, disialogangliosides and trisialogangliosides. Particularly evident was the high relative amount of a component with GM2 mobility. All of these gangliosides were also present in thymus. The main difference between thymus and bursa was the lower level of the ganglioside with GM2 mobility in the former. These patterns are in distinct contrast to those obtained with the tumors. Hematosides and bands corresponding to disialogangliosides were present in IM and IP tumors (fig. 1). A component corresponding to GM2 was also present in tumors but in much lower amounts relative to bursa and thymus. Tumors were also characterized by the almost complete absence of gangliosides with GM1 and trisialoganglioside mobilities. Thus the major differences between control tissues and the tumors were the accumulation of disialogangliosides (GD1a, GD1b and/or GD3) and diminutions of GM2, GM1 and trisialogangliosides in tumors. Ganglioside patterns in the 4 tumors examined were virtually identical.

Enzymatic activities for several of the glycosyl transferases involved in ganglioside biosynthesis are given in table 2. Only very low activities of all enzymes assayed were encountered in muscle. Thus, these data indicate that muscle contamination of IM tumors had a neglible effect on the results obtained.

Among the other tissues examined–bursa, thymus, IM and IP tumors–there were differences in activities of many of these glycosyl transferases between normal and tumor tissue (table 2). The enzyme UDP-galactose: glucosyl ceramide galactosyl transferase (lactosyl ceramide is the product) was most active in bursa and least active in thymus. IM and IP tumors had 55 and 61% of the activity of bursa with exogenous acceptor.

Both bursa and thymus had enzymes which transferred *N*-acetyl-neuraminic acid from CMP-*N*-acetyl-neuraminic acid to glycolipid acceptors. Lactosyl ceramide (product = GM3) and GM1 (product = GD1a) were more active acceptors than was GM3 (product = GD3) in both tissues. Lower levels of these sialyl transferases were present in IM and IP tumors with all acceptors. The activities in tumors amounted to from 15–40% of the activities in bursa with exogenous acceptors.

UDP-*N*-acetylgalactosamine: GM3 *N*-acetylgalactosamine transferase (product = GM2) was active in all tissues. The level of activity in tumors was approx. 60–80% of the levels in bursa. UDP-*N*-acetyl-glucosamine was inactive as a sugar donor with all tissues, indicating that gangliosides in these tissues contain galactosamine and not glucosamine.

The most pronounced diminution in enzyme activity in tumors was in levels of UDP-galactose:GM2 galactosyl transferase (GM1 is the product). Specific activities of this enzyme in IM and IP tumors were only approx. 6–10% of the level of activity in bursa. To determine whether this diminution in product

accumulation was due to the presence of an inhibitor or a glycosidase in tumors, mixing experiments were conducted. When equal quantities of bursa and IM or IP tumor total particulates were combined, specific activities were identical to those calculated for the individual enzyme sources. This indicates that the low activities in this transferase in tumors is the result of lower levels of the galactosyl transferase.

4. Discussion

Compared to the control bursal tissue, TLT tumors show no changes of great magnitude in total, bound and ganglioside sialic acid levels. As with mammalian tumors, there are distinct differences in the ganglioside pattern of TLT tumors compared to controls. The pattern changes observed in TLT tumors differs from those changes observed with other neoplastic cells and tissues [2–4, 7–9]. The general finding has been a build-up of one particular ganglioside and the absence of any higher ganglioside homologs. This is the expected result in view of the stepwise mode of assembly of the carbohydrate chain of gangliosides [25] and the finding of depression in enzyme activity at the step following the ganglioside build-up [5, 6, 9].

TLT tumors are characterized by the absence of trisialogangliosides as can be seen in fig. 1. In addition, GM2 is also diminished in amount and GM1 is almost completely absent. The net effect is that GM3 and disiaolgangliosides accumulate. The absence of trisialogangliosides can be explained by the generalized reduction in sialyl transferases observed in tumors. The accumulation of disialogangliosides is not easily explained since GM3 and GM1 were less active acceptors in tumors compared to controls. The accumulation of disialogangliosides may be due to an enzymatic block in conversion of di- to trisialoganglioside.

The reduced amount of GM2 is difficult to reconcile with the relatively high levels of *N*-acetylgalactosaminyl transferase encountered in tumors. A partial explanation could be the reduced enzyme activities at two steps prior to the formation of GM2: the conversion of glucosyl ceramide to lactosyl ceramide and then to GM3 (which in turn is converted to GM2). The virtual absence of GM1 appears to be due to the markedly lower levels of UDP-galactose:GM2 galactosyl transgerase in tumors.

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